The intricate side of systems biology

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The combination of high-throughput methods of molecular biology with advanced mathematical and computational techniques has propelled the emergent field of systems biology into a position of prominence. Unthinkable a decade ago, it has become possible to screen and analyze the expression of entire genomes, simultaneously assess large numbers of proteins and their prevalence, and characterize in detail the metabolic state of a cell population. Although very important, the focus on comprehensive networks of biological components is only one side of systems biology. Complementing large-scale assessments, and sometimes at the risk of being forgotten, are more subtle analyses that rationalize the design and functioning of biological modules in exquisite detail. This intricate side of systems biology aims at identifying the specific roles of processes and signals in smaller, fully regulated systems by computing what would happen if these signals were lacking or organized in a different fashion. We exemplify this type of approach with a detailed analysis of the regulation of glucose utilization in *Lactococcus lactis*. This organism is exposed to alternating periods of glucose availability and starvation. During starvation, it accumulates an intermediate of glycolysis, which allows it to take up glucose immediately upon availability. This notable accumulation poses a nontrivial control task that is solved with an unusual, yet ingeniously designed and timed feedforward activation system. The elucidation of this control system required high-precision, dynamic *in vivo* metabolite data, combined with methods of nonlinear systems analysis, and may serve as a paradigm for multidisciplinary approaches to fine-scaled systems biology.

biochemical systems theory | control system | feedforward activation | lactococcus lactis | metabolic pathway

As is typical with any new focus in science, the community has not yet agreed on a generally accepted definition of systems biology. Nonetheless, despite its young age, a perception is crystallizing that systems biology might be synonymous with the analysis of large networks that describe entire genomes, the totality of protein–protein interactions, the comprehensive mapping of metabolic pathway systems, or the combination of these systems at different levels of biological organization. Only a decade ago, such assemblies were unattainable both experimentally and analytically, but with modern high-throughput data acquisition techniques and ever-increasing computational power, they have come within reach. Their sheer size and high connectivity, presented with modern means of visualization, are indeed awe inspiring and have led to insights unimaginable only a few years back.

The focus on comprehensiveness is appealing, yet it would be shortsighted to make it exclusive. Biological systems are not just large, but they are organizationally complex, which, in addition to their often large numbers of components and processes, is manifest in properties like dynamics, regulation, and adaptation. These more subtle features tend to be ignored in large-scale analyses, because they create mathematical complications that presently cannot be captured or analyzed at the level of all-encompassing systems. Nevertheless, these features govern the life and responsiveness of cells and organisms in a very significant fashion, and it therefore is necessary to investigate their specific roles and functions. Because of the intrinsic complexity associated with the nonlinear dynamics or regulatory systems, it seems prudent at this point to pursue rigorous and detailed analyses of representative "sandbox examples" that help us discover successful patterns of design and operation. It is widely expected that much of biological organization is hierarchical and modular, and, if this supposition is true, insight into a variety of smaller systems will create a foundation on which to approach a deeper understanding of the functionality of large-scale integrated systems.

As an example for the intricate nature of the regulatory aspects of systems biology, we present here a model analysis of a mechanism that allows the bacterium *Lactococcus lactis* to respond very effectively to changes in glucose availability. The functionality of this regulatory mechanism is not detectable with the typical approaches of linear large-scale analysis. Instead, we demonstrate how explanations of the rationale and functioning of this controller become possible through a combination of relatively low-throughput, yet very precise, data on the dynamics of metabolic pools that were obtained through *in vivo* measurements (1), kinetic analysis by using cell extracts (2), and techniques of nonlinear systems modeling (3).

Lactococcus lactis is a member of the lactic acid bacteria widely used in the industrial manufacture of milk-fermented products. This homofermentative microorganism converts glucose (or lactose) to lactic acid, via the Embden–Meyerhof glycolytic pathway (Fig. 1), with >95% yield. The notable production of lactic acid is responsible for the protection of dairy products against spoilage by other microorganisms. In comparison with the canonical model bacteria *E. coli* and *B. subtilis*, *L. lactis* is a simpler system and, therefore, is well suited for integrative study.

NMR spectroscopy is a noninvasive technique that allows unique measurements of the kinetics of intracellular pools of metabolites directly in living cells (4). We monitored the pools of labeled intermediates and end products, with a time resolution of 30 s, in nongrowing *L. lactis* cell suspensions after a pulse of [6-^{13}C]-labeled glucose (5). In addition to lactate and glucose, the levels of fructose 1,6-bisphosphate (FBP), glucose 6-phosphate (G6P), 3-phosphoglycerate (3-PGA) and phosphoenolpyruvate (PEP) were measured online (Fig. 2).

Results and Discussion

Initial Observations and Doubts. At first glance, the data in Fig. 2 make intuitive sense. Glucose is taken up by the cells and converted into G6P and then FBP. The latter is converted into trioses (3-PGA and PEP), which ultimately form lactate. A closer look raises questions. What is the importance of such a strong, transient accumulation of FBP? What are the reasons for seemingly unimportant intermediate metabolites like 3-PGA and PEP to accumulate persistently after glucose

Conflict of interest statement: No conflicts declared.

Abbreviations: FBP, fructose 1,6-bisphosphate; G6P, glucose 6-phosphate; PEP, phosphoenolpyruvate; PK, pyruvate kinase; 3-PGA, 3-phosphoglycerate.

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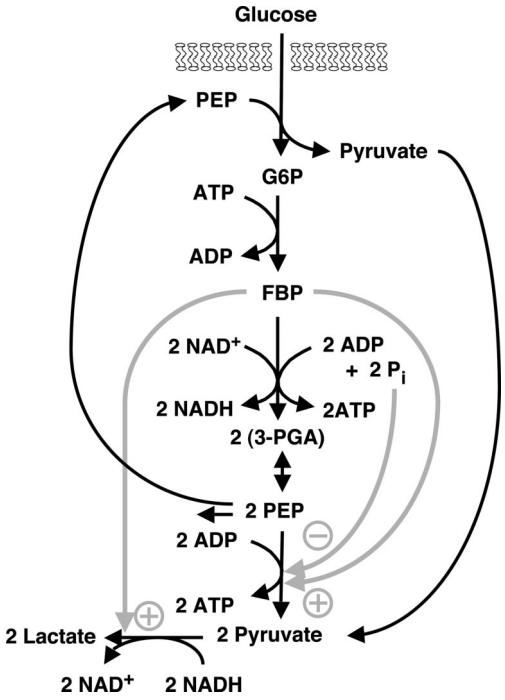

Fig. 1. Simplified representation of glycolysis and lactate production in *L. lactis*. Black arrows show flow of material. Gray arrows indicate signals, minus sign indicates inhibition, and plus signs indicate activation.

depletion? What is the functionality of the 2-fold control of the pyruvate kinase (PK) reaction that converts PEP into pyruvate?

Maybe most intriguing is the question of how glucose uptake gets started after a period of glucose starvation. Arguing based on the tenets of linear pathway analysis, any amount of available glucose would run through the glycolytic system, causing a slightly delayed, transient accumulation of all intermediates, including PEP. PEP would quickly be converted into pyruvate and, thereby, used for the production of lactate, which the bacterium releases into the medium. Furthermore, any temporary accumulation should be minimized, because the storage of otherwise unneeded intermediates is undesirable (6–8). After a period of glucose starvation, one thus would expect depletion of the PEP pool, and PEP and 3-PGA levels below the detection level at the beginning of the experiment therefore seem reasonable (*cf.* green lines in Fig. 2).

However, the apparent lack of PEP under glucose starvation would create a severe problem. In the extreme, glycolysis could not even be started, because the phosphorylation of glucose depends on phosphate, which in *Lactococcus* is primarily provided by PEP. Something must be missing in this simplistic, linear reasoning.

A more detailed analysis suggests indeed that the initial PEP pool should be of considerable size. The experimental data show that PEP and 3-PGA are at high concentration levels at the end of the experiment, and these levels apparently decrease only very slowly. Thus, imagining the time period before the present experiment, one should surmise high levels as well, resulting from the last time glucose was available and then used up (*cf.* orange lines in Fig. 2). The apparent discrepancy with the experimental data (diamonds in Fig. 2) vanishes upon realization that the NMR technique detects only labeled metabolites, with the label stemming from the glucose substrate, whereas any preexisting, unlabeled metabolite pools remain undetected. Confirming this deduction and consistent with independent

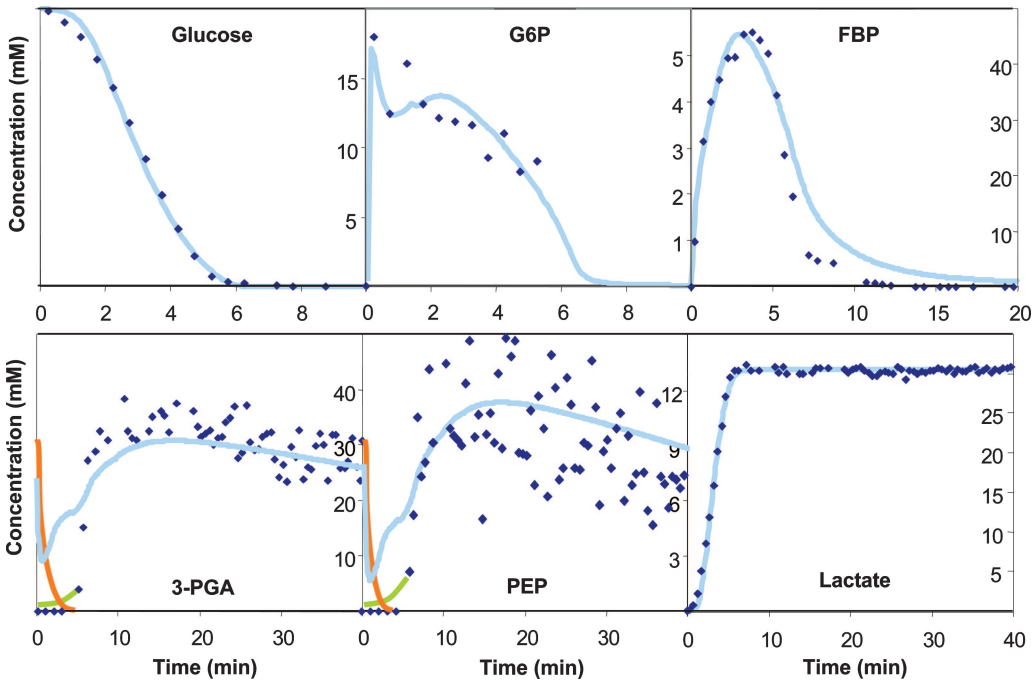

Fig. 2. Dynamics of metabolite pools in *L. lactis* strain MG1363 derived from 20 mM [6-^{13}C]glucose metabolized under aerobic conditions at pH 6.5 (5). Experimental data (dark blue diamonds) were obtained with *in vivo* ^{13}C-NMR techniques. Under the experimental conditions used, the detection limit for intracellular phosphorylated metabolites was 3 mM. Green lines, *a priori* inferred dynamics of 3-PGA and PEP below the detection level. Orange lines, dynamics of unlabeled 3-PGA and PEP, inferred from a model analysis of the observation that 3-PGA and PEP are still high in concentration after 40 min of starvation, a situation that should be similar to the beginning of the experiment. Support of this inference came from the fact that the NMR technique measures only labeled compounds but not the unlabeled 3-PGA and PEP pools at the beginning of the experiment. Light blue lines, simulation results with a mathematical model constructed under the guidelines of Biochemical Systems Theory (3, 6–9) (see *Methods* and supporting information for details).

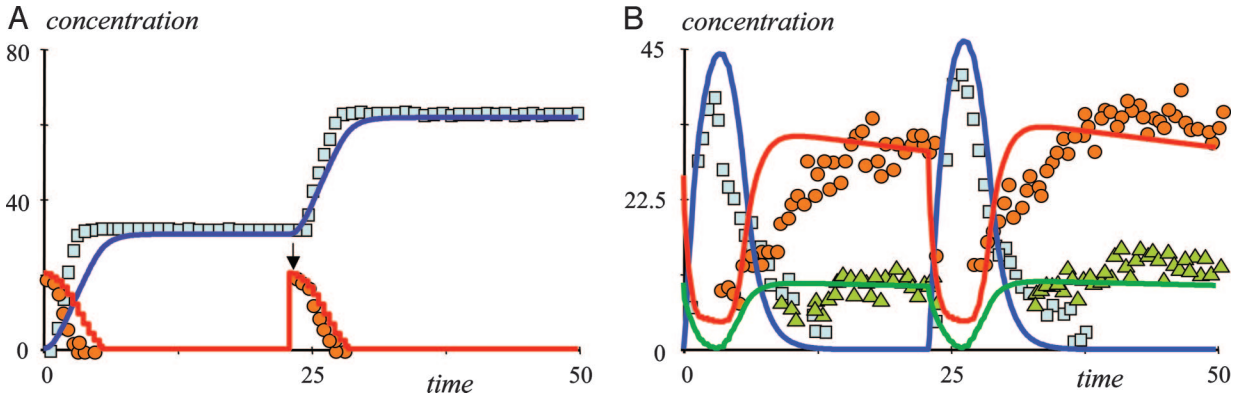

Fig. 3. Simulation of a tandem experiment (1), in which a second glucose bolus is given after 23 min (arrow in *A*). The dynamics is captured rather well, even though no parameters were readjusted. Differences seem to be caused, at least in part, by the rate of disappearance of the first bolus of glucose, which is faster than in the experimental data we used originally. (*A*) Observed dynamics of glucose (circles) and lactate (squares), superimposed with model simulation (lines). (*B*) Observed dynamics of FBP (squares), 3-PGA (circles), and PEP (triangles), superimposed with model simulation (lines). Data redrawn from ref. 1.

observations (2, 9), an experiment with the addition of a second, identical pulse of labeled glucose resulted in large pools of labeled 3-PGA and PEP that derived from the first pulse of glucose and decreased to undetectable levels within seconds upon the second glucose supply (ref. 1; Fig. 3). Our conclusion therefore is that the trioses 3-PGA and PEP should have high values at the beginning of the experiments (comparable with those at the end of the experiment), whereas G6P and FBP would be essentially depleted, as observed. The unlabeled triose pools would be consumed rapidly during glucose phosphorylation (orange lines in Fig. 2).

To test the validity of this conclusion, we simulated the tandem experiment (1) with our model by using exactly the same parameter values as before. As in the wet experiment, a second pulse of glucose was supplied at 23 min, and the offline dynamics of ATP and P_i was assumed to repeat itself as at the beginning of the experiment. Even without any reparameterization, the responses of the model reflect the actual observations quite well (Fig. 3). One notes that the glucose degradation in this experiment is slightly different from our previous experiments (Fig. 3*A*). This difference is the consequence of interexperimental variability and is not a fitting error, because the glucose data of the original experiment enter the model as offline input, and these data are used here again. As secondary consequences, the lactate dynamics in the tandem model is slower than observed (Fig. 3*A*), and the dynamics of FBP and 3-PGA in the model is faster, leading to 10–15% higher peak values in FBP (Fig. 3*B*). Interestingly, in both the observation and the model, the second FBP peak is slightly higher than the first, which one may interpret as a consequence of the residual amounts of 3-PGA and PEP, which are somewhat higher at $t = 23$ than at $t = 0$. The first data points of 3-PGA and PEP after the second glucose bolus are, without any intervention, very accurately predicted by the model (Fig. 3*B*).

These simulations and conclusions raise the interesting question of how the cell manages to achieve and maintain a high PEP concentration under conditions of glucose starvation. As argued before, a pathway model without regulation hardly would be able to explain this phenomenon. Regulation provides a possible explanation, according to the following hypothesized scenario. FBP has been shown to be a strong activator of PK (9). With FBP decreasing toward zero and the inhibitor P_i increasing beyond 20 mM, PK is no longer active, and PEP can no longer be converted into pyruvate. The second pathway of PEP dephosphorylation, the glucose transport system, becomes limited by glucose availability at this stage. Thus, PEP is "trapped." Because PEP and 3-PGA are related through a reversible reaction, 3-PGA also reaches a more or less constant level that is in the observed equilibrium with PEP. It is interesting to note that the noise in PEP and 3-PGA at these later stages of the experiment is much higher than in all other metabolite pools of the system, which may be an indication of the intense shuttling between the two pools.

Analysis per Simplified Model. It is easy to arrive at faulty conclusions when using hand waving arguments. Therefore, to assess the validity and efficacy of the proposed mechanism of starting and stopping glycolysis, while not being distracted by details and uncertainties of the true pathway, we mimicked the unusual feedforward activation system with a simplified linear pathway in which uptake of substrate requires a downstream metabolite (X_4) as a second substrate, activator, or cofactor and where an early metabolite (X_2) activates the degradation of X_4 (Fig. 4). Thus, X_1 corresponds to G6P, X_2 to FBP, X_3 to 3-PGA, X_4 to PEP, and X_5 to pyruvate.

With typical parameter values, which are numerically not sensitive, a model of the pathway may read

$$\begin{aligned}
\dot{X}_1 &= Input_1 + Input_2 \cdot X_4^{0.5} - X_1^{0.5} && X_1(0) = 1,\\
\dot{X}_2 &= X_1^{0.5} - X_2^{0.75} && X_2(0) = 1,\\
\dot{X}_3 &= 2X_2^{0.75} - 2X_3^{0.4} && X_3(0) = 1,\\
\dot{X}_4 &= 2X_3^{0.4} - Input_2 \cdot X_4^{0.5} - X_2^{h_{42}} X_4^{0.5} && X_4(0) = 1, \text{ and}\\
\dot{X}_5 &= X_2^{h_{42}} X_4^{0.5} - X_5^{0.5} && X_5(0) = 1.
\end{aligned} \qquad [1]$$

The system has two inputs, one that represents some low-level constant substrate supply ($Input_1 = 0.01$), which prevents the system dynamics from ceasing altogether, whereas the other one ($Input_2 = 0.99$) is controlled by X_4. In the *Lactococcus* system, these inputs correspond to ATP-based and PTS-based glucose phosphorylation, respectively. The steady state of the system is $(1, 1, \ldots, 1)$. The parameter h_{42} in the conversion of X_4 into X_5 reflects the feedforward activation of primary interest. With the value $h_{42} = 0.75$, the system shows the response in Fig. 5*A*. For this time course, the system starts at its steady state. At $t = 10$

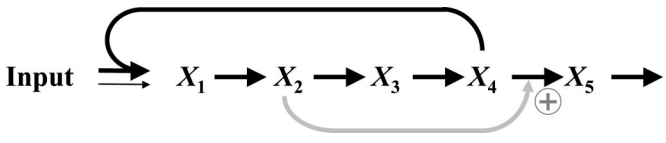

Fig. 4. Generic linear feedforward activated pathway in which a downstream metabolite (X_4) is needed as a second substrate for the first step.

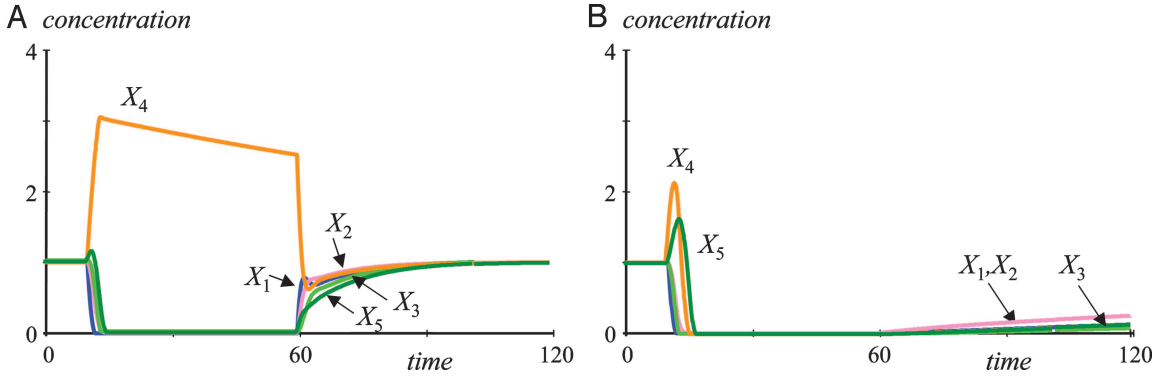

Fig. 5. Responses of the pathway in Fig. 4, as implemented in Eq. **1**, where the main substrate influx is stopped between $t = 10$ and $t = 60$. (*A*) X_2 activates the degradation of X_4. (*B*) The activation of the degradation of X_4 by X_2 is eliminated.

min, the main input is suddenly stopped ($Input_2 = 0$). Because of this artificial suddenness, all variables except for X_4 almost immediately approach a very low value that is maintained by the constant low-level input. With X_2 close to zero, its activation of the degradation of X_4 ceases, and X_4 is produced but virtually not degraded. In comparison with the *Lactococcus* data, the dynamics of X_4 does not show the initial decrease. This difference in response is due to simplifications in the artificial pathway. Of main importance is that upon restoration of $Input_2$ at $t = 60$, the system is immediately ready to resume activity.

The feedforward activation design is to be compared with an otherwise equivalent pathway with $h_{42} = 0$, which means that X_2 does not feed information forward to the degradation of X_4. Everything else remains the same. It is noted that this type of analysis would be difficult to execute experimentally, because one would have to create a pyruvate kinase that is insensitive to FBP activation, without having any other metabolic consequences. The model response now is strikingly different (Fig. 5*B*). Because the lack of X_2 does not stop the degradation of X_4, X_4 decreases in sequence with all other metabolites. As soon as the substrate input is restored ($t = 60$), the low residual amounts of X_4 allow the system to take up the substrate, but only at a very slow rate, and with the given parameter values, it takes ≈30 h before the pathway is within 5% of its original steady state.

In the *Lactococcus* pathway, the reaction between 3-PGA and PEP is reversible. Including this reversibility in the simplified model does not change our conclusions. In fact, even with a reverse flux one-third as strong as the forward flux, the quantitative results are almost indistinguishable, except for a slightly decreased peak level of X_4 and a residual amount of X_3 during starvation (see supporting information, which is published on the PNAS web site).

One also could surmise that more gradual changes in input might alter our conclusions on the effect of feedforward activation. To test this proposition, we ramped the input down during the time period between 10 and 20 min and ramped it up during the time period between 60 and 70 min. As expected, the dynamic responses of the model system were "softened" by this change, but the generic conclusions remained unchanged (see supporting information).

Implications for *Lactococcus*. If the interpretations from the simplified model hold for *Lactococcus*, the feedforward activation of the pyruvate kinase reaction by FBP translates any lack of glucose into a stop of glycolysis at the perfect position, namely the one that would otherwise use up PEP. By doing so, the mechanism creates a holding pattern that is immediately ready to take up new glucose. To test this hypothesis, we used our model of glycolysis in *Lactococcus*. This model indeed captures the observed and inferred data well (light blue lines in Fig. 2; see supporting information for details) and, in particular, confirms the efficacy of the stop-and-hold mechanism that we proposed from the simplified artificial pathway above.

Interestingly from the viewpoint of network design is that strong FBP activation would suffice to control glucose uptake in a desirable manner, but that PK is additionally inhibited by inorganic phosphate (P_i; see Fig. 1). Indeed, Mason *et al.* (2) experimentally investigated this feedforward inhibition in the same organism and argued that P_i rather than FBP controlled glucose uptake. At first glance, activation by FBP and inhibition by P_i seem interchangeable, because the dynamics of P_i essentially exhibits the upside-down image of the dynamics of FBP (compare Figs. 2 and 6). However, there are significant strategic differences between FBP or P_i controlling glycolysis. First, all available data indicate that P_i drops very quickly upon glucose availability. Without FBP control, this drop would imply a very fast release of PK inhibition and could lead to competition between PK and PTS that would be undesirable in this early phase of glucose utilization. More importantly, the dynamics of P_i is coupled to a large number of reactions, including many of those that involve ADP and ATP. Thus, whereas control by FBP is specific, control by P_i would allow a variety of other metabolic processes to affect glycolysis in a very significant fashion. Without FBP control, any drop in P_i during glucose starvation would lead to an immediate depletion of the PEP pool. For example,

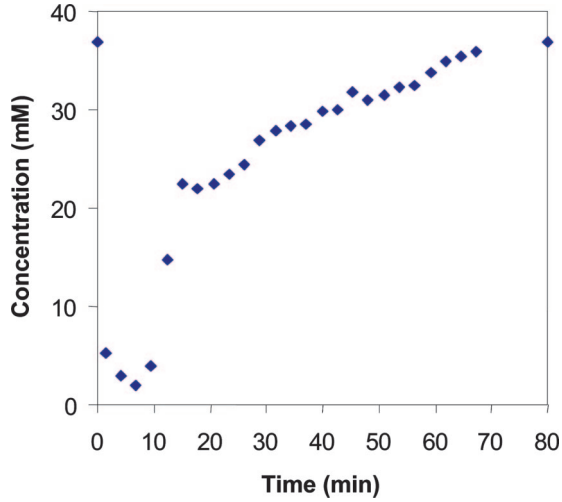

Fig. 6. Dynamics of inorganic phosphate during lactate production in *L. lactis*. Measurements were obtained with *in vivo* NMR techniques (5).

Mason *et al.* (2) found that the P_i dynamics after a bolus of glucose depends dramatically on temperature. At 15°C, the P_i level remains low, whereas it returns rather quickly to a high level at 30°C. If P_i were the main controller of PK activity, temperature in effect would become a regulator of glucose uptake. Furthermore, the return to high P_i levels is only possible through reactions outside glycolysis, because glycolysis is a P_i sink. Taken together, any regulation by P_i without FBP control would be delegated to other mechanisms and become one step removed from the target action.

The model allowed us to test deductions on the relative importance of FBP and P_i. Specifically, we performed simulations where we compared the responses of our *Lactococcus* model with alternative models where PK was unregulated, only activated by FBP or only inhibited by P_i (see supporting information for numerical results). As to be expected, the unregulated model showed sequential increases and decreases in all glycolytic metabolites after the initial few minutes when "old" PEP still was available, and only lactate accumulated toward the end. Of particular note, no PEP remained in the system to restart glucose utilization. Confirming our results with the artificial, simplified pathway (Figs. 4 and 5), the FBP-activated model showed essentially the same responses as the fully regulated model. By contrast, the P_i-controlled model did not lead to PEP and 3-PGA accumulation toward the end of the experiment, even if we altered the strength of P_i inhibition up or down. Further analysis suggested the dynamics of P_i as the main reason. This dynamics, although being more or less the mirror image of FBP (*cf.* Figs. 2 and 6), is delayed by between 5 and 10 minutes. This delay in the resurgence of P_i is crucial, because it is sufficient for FBP to deplete before the inhibition of PK causes PEP and 3-PGA to accumulate. Thus, in addition to the general strategic considerations above, the numerical features of the dynamics of the system support the importance of FBP as a crucial controller of the pathway.

Not shown in Fig. 1 is that FBP actually is in equilibrium with DHAP and Ga3P and that PEP is in equilibrium with 3-PGA and 2-PGA. These sets of readily interchangeable pools may have their own functions, but the reversible steps also contribute to a slight time delay and to a softening of the role of FBP as terminator of glycolysis. While material along the glycolytic pathway between FBP and PEP is still being processed and ultimately accumulates in PEP and 3-PGA, they give the system enough time between shutting down PK and replenishing PEP to the observed "holding" level. Because the reaction between PEP and 3-PGA is reversible, 3-PGA (as well as other related trioses) must be retained at normal levels also, otherwise PEP would drain from the system. Eventually, these pools do leak, possibly due to residual PK activity, which may be needed for the low-level generation of pyruvate and other critical compounds.

One should note that Mason *et al.* (2) came to the conclusion that P_i, rather than FBP, should be the dominant controller of glycolysis, because PK can be active in the absence of FBP. However, this activity only happens for P_i concentrations $<\approx 3$ or 4 mM, which, in our case, only occur very briefly (*cf.* Fig. 6). The same data reveal approximately two-thirds maximal PK activity if FBP is present at a physiological level of 30 mM, even if P_i is at a relatively high concentration (20 mM). This finding suggests that, for physiological P_i concentrations, PK is active only if FBP is present in sufficiently high concentrations. These quantitative considerations also show that the control mechanism is rather insensitive to physiological fluctuations in P_i and prevents PK from catalyzing PEP unless P_i is really low and FBP is very high. One explanation for the role of P_i, rather than being a direct effector, may be that even low P_i values increase considerably the K_a for FBP, thereby further increasing its regulating effect as activator (10–11).

Conclusions

It is usually assumed that the accumulation of intermediates in a linear pathway is disadvantageous, because their storage is unnecessary yet chemically costly (6–8). The observation of high 3-PGA and PEP pools in *Lactococcus* therefore appears to point to a suboptimal pathway design. However, closer scrutiny corrects this conclusion. Many homofermentative lactic acid bacteria, including *Lactococcus*, live in environments where glucose availability may fluctuate widely between high concentrations and extended periods of starvation. As long as glucose is plentiful, the bacteria employ efficient transporters that feed the substrate into metabolic pathways that, in turn, use it for energy production and population growth. In situations without sugars in the medium, the organisms cannot grow. During these periods of starvation, it becomes crucial to be well prepared for future availability of glucose, which the organisms must use quickly for energy generation and for the excretion of lactate, whose acidity helps them create and maintain an advantage over potential competitors. To achieve this readiness, the organisms must enter a holding pattern that is characterized by high concentrations of PEP, which is needed as phosphate donor for glucose consumption.

Without an effective control design, a holding pattern of this type would not be possible. All glucose would be converted into lactate and other end products, such as acetate, acetoin, and ethanol. In particular, without PEP, *Lactococcus* would be less competitive against organisms by using ATP for glucose phosphorylation and any sudden availability of glucose would be of little benefit, because of the organism's sluggishness in taking up the substrate. *Lactococcus* and other homofermentative lactic acid bacteria therefore are faced with the design task of maintaining PEP at relatively high concentration levels that last long enough to bridge normal periods of starvation. It appears that these concentration levels are fine tuned so that the amount of phosphate donors necessary for rapid glucose utilization after starvation are just sufficient to hold over until *de novo* trioses are provided through glycolysis.

Our comparative studies with lacking FBP and/or P_i regulation demonstrate the power of detailed systems biological analyses. Experimentally, such studies are very difficult, if not impossible (12), but their computational equivalents are easily capable of deciphering the advantages of one regulatory design over another. In the present case, they show that accomplishing the goal or retaining PEP not only requires a clever structural and regulatory design but also critically depends on precise timing. If the outlet of the pathway, catalyzed by pyruvate kinase, is closed too rapidly, unnecessary amounts of material are stored in the form of trioses. Otherwise, if pyruvate kinase is deactivated too slowly, most glycolytic material is converted into lactate, thereby causing PEP depletion that is detrimental for future glucose utilization.

Lactococcus operates the glycolytic pathway and its needed PEP holding pattern with a feedforward activation mechanism that is rare in metabolic systems. This mechanism is fortified with a second mechanism of feedforward inhibition by P_i that, by itself, appears to be inferior. The observed design, when elucidated in this fashion, proves to be very effective. The strong transient peak of FBP (Fig. 2) facilitates a very quick conversion of PEP into pyruvate and lactate while glucose is available but is also an effective stop of PK activity when glucose is no longer available. The source and position of activation appear to be optimal. In contrast to G6P, which is a major metabolic branch and control point, and to F6P, which is in very fast equilibrium with G6P, FBP is the first intermediate dedicated to glycolysis but not much else. By virtue of the fact that FBP activates PK, and not some other intermediate step, glycolysis stops and holds at the perfect position, namely PEP.

The regulation by FBP is accompanied by a secondary regulatory mechanism involving P_i. Without FBP, this regulation would be

sensitive to P_i fluctuations anywhere in the cell, thereby disqualifying P_i as sole regulator. Such P_i fluctuations are frequent occurrences, because they are connected with many changes in the energy status of the cell that, in turn, affects the activity of glycolysis, because PFK is activated by ADP. Under normal conditions, P_i inhibition and FBP activation have complementary roles, and P_i therefore solidifies the start-and-hold mechanism controlling glucose utilization. An interesting detail is the surprisingly large pool of FBP that accumulates transiently during glucose consumption. Although there is no experimental proof, one may surmise its role as a protectant against situations where glucose is available, but P_i is high for extraneous reasons, for instance, because large quantities of ATP had been used somewhere in the cell. The high P_i level would inhibit PK, thereby leading to an accumulation of trioses at an inopportune time that would be controlled by factors outside glycolysis. Only a strong peak in FBP would overcome this incidentally inappropriate control.

One may speculate why *Lactococcus* uses the PTS system rather than ATP for glucose phosphorylation. Despite the clear disadvantage of a strong dependence on a well timed, reliable PEP dynamics, this design has the notable advantage for *Lactococcus* that most of the glycolytic process is short-circuited through the PTS system. Thus, the organism uses the first available glucose directly to produce pyruvate and then lactate, thereby souring the surrounding medium at a critical time when potential competitors attempt to take up glucose. Of note is that this process is independent of FBP, which at that point is still in its depleted state.

The mechanism of feedforward activation is unusual. Feedback inhibition is widely recognized as a ubiquitous mechanism of controlling the sizes of metabolite pools. Feedforward inhibition has been observed in a number of pathway systems and, under the right conditions, can have a stabilizing effect on the pathway (13–14). Feedback activation usually is dreaded, because it often leads to instability. Feedforward activation has been reported in neuronal systems, but hardly in a metabolic context, and activation of PK by FBP is indeed the best-known example. We have shown here that feedforward activation, properly embedded in a regulatory system, provides a potent tool of pathway control.

Stepping back from these pathway-specific features, our analysis demonstrates how important it is to investigate the timing and the regulatory features within a system in intricate detail. Such investigations are possible only if they are based on data that are obtained under physiological conditions and that are crisp enough to permit differentiating analyses. Combining such data with nonlinear dynamic analyses seems to be the most promising path toward discovering natural design principles and developing a true understanding of complex systems in biology.

Methods: Model Design

Directly based on the diagram in Fig. 1, we constructed and implemented a series of fully kinetic pathway models within Biochemical Systems Theory (3, 6, 15–17) as modeling framework. Choosing the Generalized Mass Action representation, the resulting model in symbolic form is

$$\begin{aligned}
X_1 &= \textit{Offline Glucose Input},\\
ATP &= \textit{Offline Concentration of ATP},\\
P_i &= \textit{Offline Concentration of } P_i\\
\dot{X}_2 &= \alpha_2 X_1^{g21} X_2^{g22} X_5^{g25} - \beta_2 X_2^{h22} ATP^{h2,ATP},\\
\dot{X}_3 &= \beta_2 X_2^{h22} ATP^{h2,ATP} - \beta_3 X_3^{h33} P_i^{h3,Pi},\\
\dot{X}_4 &= 2\beta_3 X_3^{h33} P_i^{h3,pi} + \alpha_4 X_5^{g45} - \beta_4 X_4^{h44},\\
\dot{X}_5 &= \beta_4 X_4^{h44} - \alpha_2 X_1^{g21} X_2^{g22} X_5^{g25} - \alpha_4 X_5^{g45}\\
&\quad - \beta_{51} X_5^{h513} X_5^{h515} P_i^{h51,Pi} - \beta_{52} X_5^{h525},\\
\dot{X}_6 &= \alpha_2 X_1^{g21} X_2^{g22} X_5^{g25} + \beta_{51} X_3^{h513} X_5^{h515} P_i^{h51,Pi}\\
&\quad - \beta_{61} X_3^{h613} X_6^{h616} - \beta_{62} X_6^{h626},\\
\dot{X}_7 &= \beta_{61} X_3^{h613} X_6^{h616}.
\end{aligned} \qquad [2]$$

Although setting up the equations was straightforward for most variables, ATP and P_i were problematic, because these two variables are involved in many reactions that cannot all be included in the model. ATP and P_i, therefore, were not modeled as differential equations but as "offline" input functions in the form of raw data that were locally smoothed (18–19). Similarly, glucose was represented as an offline function, because the observed glucose uptake follows a sigmoidal function that is incompatible with the structure of the pathway (18). NAD and NADH were essentially constant under the aerobic conditions modeled here (data not shown). Their concentrations therefore were subsumed into the corresponding rate constants. The production term of G6P contains G6P itself, because it could be that G6P inhibits glucose uptake and utilization (20), even though this mechanism has not been demonstrated for *L. lactis*.

Parameter values were obtained from the observed time course data (Fig. 2) with a combination of inverse methods, as described elsewhere *in extenso* (18). Pertinent numerical results and technical details of model implementation in the freely available PLAS (www.dqb.fc.ul.pt/docentes/aferreira/plas.html) are given in supporting information.

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